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WITHOUT THE USE OF INERT GASES

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SOME DEVICES

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CULTIVATION OF ANAËROBIC BACTERIA IN FLUID MEDIA WITHOUT THE USE OF INERT GASES.

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The multiplication of anaërobic bacteria in bouillon from which the air is not wholly excluded was first pointed out by Kitt[1] in his study of the bacillus of Rauschbrand. It had been noted by various observers before Kitt that anaërobic bacteria will multiply and produce spores on the inclined surface of ordinary cotton-plugged agar tubes when they are mingled with aërobes; in other words, when the culture is a mixed or impure one. Within the past few years the associated growth of anaërobes and aërobes in culture media freely exposed to the air has been the subject of a number of publications. It is not the object of this paper to enter into a discussion of the literature or the various hypotheses which have been brought forward to explain anaërobiosis, but simply to present a few devices which I have resorted to in the cultivation of anaërobic bacteria, more particularly the tetanus bacillus, for the production of a satisfactory tetanus toxin. The tetanus bacillus, like the bacillus of diphtheria, produces a toxin which diffuses into the culture fluid. When the bacteria have been removed by filtration the filtrate is still highly toxic and may be employed to immunize horses destined to yield antitoxin. The cultivation of the tetanus bacillus in fluids on a large scale for this purpose demanded apparatus simple and easily manipulated, to reduce as far as possible the danger associated with the manipulation. The removal of the air by aspiration, and the substitution of

[1] Centralblatt f. Bakteriologie, xvii, s. 168.

hydrogen, is a procedure requiring much time, and, especially with fluid cultures, is likely to lead to unsuspected contamination with miscellaneous bacteria.

The development of anaërobes in the ordinary fermentation tube was known to me for some years, and I set about devising culture flasks which would imitate this. The fermentation tube is, however, not fully satisfactory, because the contact of the fluid with the air is a little too free for some anaërobes. The multiplication does not go on at times as freely as is desirable unless the culture fluid is thoroughly steamed before inoculation. The bacilli may degenerate and fail to sporulate.

The culture apparatus shown in Figure 1 was exhibited to this Society in 1897, but no description published. It consists of two bulbs, *a* and *b*, which are of nearly equal capacity, and which may be made as large as desired. The two are connected by means of a heavy rubber tube *c*. A clamp *d* is of use at times to restrict communication between *a* and *b* to a minimum. The bouillon occupies the whole of *a*, the connecting tube, and all below the dotted line in *b*. It is inoculated by transferring material containing the bacillus or its spores with platinum loop or pipette through the cotton-plugged opening *e*. The growth travels down into the bulb *a* within twenty-four hours. Bulb *a* may be constructed with an upper opening to facilitate filling and cleansing. Such opening must be kept tightly stoppered and the rubber stopper wired down so that air may be absolutely excluded. It is evident that the bulb *b* is needed to receive the fluid almost wholly driven out of *a* by steam forming in it during sterilization, as well as the fluid crowded out of *a* by gases which are quite regularly produced by anaërobes in media containing dextrose and some other sugars. The apparatus is best manipulated in a rack of tin *f*, easily made for it.

For more than a year past I have replaced the culture apparatus just described by a flask in some respects more, in others less, satisfactory. This is shown in Figure 2. It consists of a stout litre flask *a* into which is fitted, with the aid of a rubber stopper *c*, a 100 c.c. pipette *b*, with the lower por-

PLATE II.

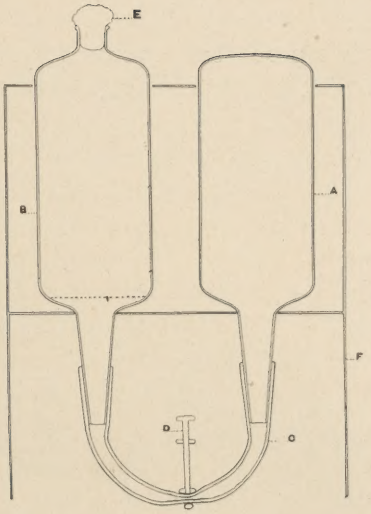

FIG. 1.

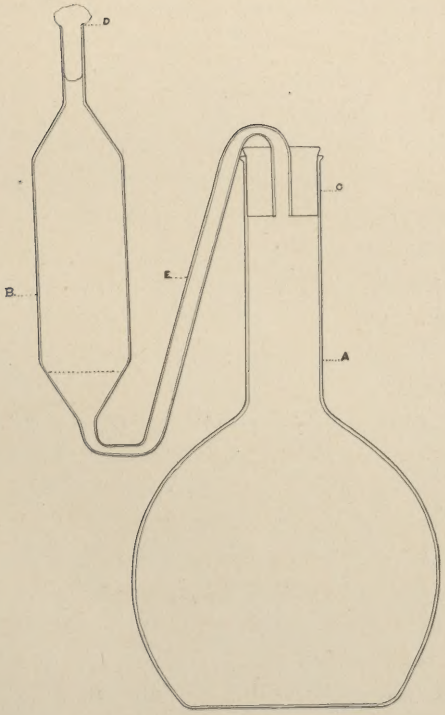

FIG. 2.

tion *e* bent as shown in the figure[1] and the upper shortened and provided with a cotton plug *d*. The bouillon fills the flask completely and extends down the narrow tube to the dotted line in the bulb. It is inoculated through the opening *d*. The growth proceeds unaided along the narrow tube and reaches the flask in twenty-four to thirty-six hours.

These two flasks permit a rich growth of the tetanus bacillus. In about five or six days the multiplication ceases. With the culture in my hands the unfiltered bouillon is fatal to a guinea-pig in doses of .00005 c.c. to .0002 c.c.[2]

The second flask has one disadvantage which necessitates some additional work. It cannot be autoclaved when filled, because the sudden ebullition throws out some of the fluid. In the Arnold sterilizer this does not occur and no difficulty is experienced. To obviate the loss of fluid in the autoclave the following procedure has been adopted: The flask itself is not entirely filled, but as much as is needed for this purpose is autoclaved with it in an ordinary flask. During the sterilization the bulb *b* with attached stopper either rests lightly in the flask or may be placed separately into the autoclave. After the sterilization the flask is filled up with the extra fluid, and the stopper forced in and tied down with twine. This can be done without contaminating the contents. In any case a final steaming in the Arnold sterilizer proves successful. If the flask is simply steamed it must be carefully watched, as the undestroyed spores of anaërobes may germinate after the third or even the fourth steaming. It is best to steam the flask in the late afternoon and place it into the incubator. Early next morning the flask should be steamed again and kept during the night in the incubator. During the next day the third steaming should take place. Finally, after an incubation of forty-eight hours, the flask is steamed for the last time. Even with the most careful watching, flasks

[1] The angle formed by the bulb *b* and the ascending tube *e* must be at least as great as drawn in the figure. Should any air bubbles remain in the upper bend of *e* after sterilization they can be tilted out without wetting the cotton plug *d*. If the angle is too small the fluid will run out at *d* when the flask is inclined to remove such bubbles.

[2] The bouillon here referred to is the ordinary peptone bouillon prepared from fresh beef. Sugar should not be added unless the specific action on different sugars is to be studied. The beef contains enough dextrose to promote multiplication in these flasks.

may suddenly become turbid over night. In all cases the offending organism is an anaërobic bacillus. The difficulty with which the spores of these bacilli germinate is only equalled by the rapidity with which they multiply when once started. It is obvious from what has been stated that the autoclave is to be preferred in the sterilization of not only this flask, but of culture fluids in general.[1]

A third device which has rendered valuable aid in continuing the cultivation of anaërobes is the following: A guinea-pig, rabbit, pigeon, or other small animal is killed with chloroform, and pieces of the internal organs, more particularly the spleen, liver, and kidneys, as large as peas or beans, are torn quickly from the organs with sterile forceps and introduced into fermentation tubes containing ordinary peptone bouillon. The tissue should be eventually forced into the closed branch of the tube with a stout platinum wire. A series of such tubes are prepared at one time and placed in the incubator for several days to reveal any contaminating bacteria from the air or the introduced tissue. Tubes provided in this way with bits of sterile tissues furnish most favorable conditions for the cultivation of anaërobes. They may be kept indefinitely and when partially dried out, refilled with sterile water. Anaërobes will still multiply freely in them, though they have not been reboiled. In fact, boiling would cloud the bouillon by coagulating the albumen from the introduced tissue. I have obtained in these tubes most copious spore-production of the tetanus bacillus. It is frequently desirable to have on hand fluid cultures of this and other anaërobic bacilli for the study of morphological and physiological characters. The simple method suggested enables us to obtain them at any time as readily as the commonly employed but much less accessible and less convenient deep-tube cultures in solid media.

[1] See also my paper on this subject in the Journal of Experimental Medicine, iii (1898), p. 647.